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ARTICLE

# Gametogenesis and Assessment of Nonlethal Tools to Assign Sex and Reproductive Condition in Burbot

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***Abstract***

**Nonlethal tools (plasma sex steroid concentrations and ultrasound) for assigning sex and reproductive condition in Burbot *Lota lota* from Lake Roosevelt, Washington, were assessed. Gonadal tissue, blood plasma, and gonadal sonograms were collected monthly from November 2016 to March 2018. Gametogenesis was described by gonadal histology during an entire reproductive cycle to confirm sex and reproductive condition. Plasma testosterone (T) and estradiol-17β (E2) concentrations were measured by radioimmunoassay. Plasma 11-ketotestosterone (11-KT) concentrations were measured by liquid chromatography–mass spectrometry. Plasma sex steroid profiles, gonadosomatic index, and ovarian follicle diameter were also described during an entire reproductive cycle. Plasma 11-KT concentration was used to assign sex with 82% accuracy during the entire reproductive cycle, and plasma 11-KT and E2 concentrations were used to assign sex with 98% accuracy when fish were reproductive (i.e., November–March in Lake Roosevelt). Plasma T and E2 concentrations were used to assign reproductive condition in females with 98% accuracy, and plasma T concentration was used to assign reproductive condition in males with 90% accuracy. Ultrasound was used to assign sex with 96% accuracy but was not useful for assigning reproductive condition. Nonlethal tools to assign sex and reproductive condition will enable fisheries biologists to assess reproductive indices of the Burbot population in Lake Roosevelt to inform management decisions.**

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Fisheries biologists need to determine sex and reproductive condition of fish in order to assess reproductive indices (e.g., sex ratio, reproductive structure, and spawning periodicity) of a population. Reproductive indices are used to characterize and monitor population demographics, model population growth, establish sustainable harvest regulations, and monitor the effects of management actions and environmental stressors (Trippel 1993; Downs et al. 1997; Power 2007; Wildhaber et al. 2007; Morgan 2008; Schill et al. 2010). Despite the utility of reproductive indices for fisheries management and conservation, they are often difficult to assess because they require extensive effort and sacrificing fish (e.g., gonadal histology). Sacrificing fish may not be preferable or possible; therefore, fisheries biologists have sought nonlethal tools to assess reproductive indices.

Plasma sex steroids have been used to assign sex and reproductive condition in modern teleosts, such as the Arapaima *Arapaima gigas* (Chu-Koo et al. 2009), Hapuka *Polyprion oxygeneios* (Kohn et al. 2013), Cutthroat Trout *Oncorhynchus clarkii* (Bangs and Nagler 2014), and Southern Flounder *Paralichthys lethostigma* (Grieshaber et al. 2016), and in phylogenetically ancient sturgeons, including the Stellate Sturgeon *Acipenser stellatus* (Ceapa et al. 2002), White Sturgeon *Acipenser transmontanus* (Webb et al. 2002; Feist et al. 2004), Persian Sturgeon *Acipenser persicus* (Viayeh et al. 2006), Lake Sturgeon *Acipenser fulvescens* (Craig et al. 2009), and Chinese Sturgeon *Acipenser sinensis* (Du et al. 2017). During gonadal development, plasma sex steroids have distinct patterns of synthesis known as plasma sex steroid profiles. For example, in female Rainbow Trout *O. mykiss*, plasma testosterone (T) and estradiol-17β (E2) concentrations peak prior to ovulation, with plasma T concentrations being much higher than plasma E2 concentrations (Scott et al. 1980a). Plasma 11-ketotestosterone (11-KT) concentrations also peak prior to ovulation but are much lower than plasma T and E2 concentrations (Scott et al. 1980a). During ovulation, plasma T concentrations are elevated, whereas plasma E2 concentrations are low or nondetectable (Scott et al. 1980a). In male Rainbow Trout, plasma T concentrations increase until spermiation begins, while plasma 11-KT concentrations continue to increase until spermiation peaks (Scott et al. 1980b). Plasma E2 concentrations in male Rainbow Trout remain low throughout the reproductive cycle (Scott et al. 1980b). Describing the plasma sex steroid profiles for a population may enable individual sex and reproductive condition to be assigned by quantifying plasma sex steroid concentrations if those concentrations differ among assignments.

Ultrasound has been used to assign sex and reproductive condition in fishes, such as the Atlantic Cod *Gadus morhua* (Karlsen and Holm 1994), Stellate Sturgeon (Moghim et al. 2002), Murray Cod *Maccullochella peelii* (Newman et al. 2008), Pacific Halibut *Hippoglossus stenolepis* (Loher and Stephens 2011), Cutthroat Trout (Bangs and Nagler 2014), and Hapuka (Kohn et al. 2013). Ultrasound can be used to accurately assign sex because of the gross morphological differences between ovaries and testes in fish (Moghim et al. 2002; Loher and Stephens 2011). For example, sex can be assigned with 100% accuracy in Pacific Halibut because the ovaries are rounded and tapered, whereas the testes are sickle-shaped and pointed (Loher and Stephens 2011). Ultrasound has also been used to assign reproductive condition by detecting visual changes in developing gonads (e.g., appearance of oocytes, changes in tissue echogenicity, and differences in gonad size; Evans et al. 2004; Novelo and Tiersch 2016). In female steelhead (anadromous Rainbow Trout), ultrasound was used to detect morphological differences between prespawn and postspawn ovaries (Evans et al. 2004). Prespawn ovaries had many ovarian follicles, whereas postspawn ovaries had only remnant ovarian follicles (Evans et al. 2004). In male steelhead, ultrasound was used to measure differences in gonad size between prespawn and postspawn testes. Prespawn testes had a mean area of 2.86 $cm^2$, whereas postspawn testes had a mean area of 0.62 $cm^2$ (Evans et al. 2004). An ultrasound index of gonadal development in female Channel Catfish *Ictalurus punctatus* was also described using morphological and echogenic changes in ovaries during development (Novelo and Tiersch 2016). Undeveloped (i.e., nonreproductive) ovaries were differentiated by homogeneous echogenic texture and a lack of distinct oocyte structures, whereas developing (i.e., reproductive) ovaries had distinct oocyte structures (Novelo and Tiersch 2016).

Burbot *Lota lota* have synchronous gonadal development and do not exhibit sexual dimorphism (Cott et al. 2013), making it difficult for fisheries biologists to assess reproductive indices in this species. Sex and reproductive condition are only evident when Burbot release gametes. Nonlethal tools, such as plasma sex steroid concentrations and ultrasound, would enable fisheries biologists to assess reproductive indices of Burbot without sacrificing the fish. To validate nonlethal tools, gonadal histology must be used to determine sex and reproductive condition, as it is considered the most accurate method of doing so (Blazer 2002). However, gametogenesis in Burbot has never been fully described by gonadal histology, with only a single study describing portions of gametogenesis (Schaefer et al. 2016). Without a description of gametogenesis, gonadal histology cannot be used to validate nonlethal tools for assigning sex and reproductive condition in Burbot.

The objectives of this study were to (1) describe gametogenesis and other indicators of maturity (e.g., gonadosomatic index [GSI], ovarian follicle diameter, and plasma sex steroid profiles) in Burbot and (2) assess nonlethal tools (plasma sex steroid concentrations and ultrasound)

to assign sex and reproductive condition in Burbot from Lake Roosevelt, Washington. The availability of nonlethal tools for assigning sex and reproductive condition will enable fisheries biologists to assess reproductive indices of the Burbot population in Lake Roosevelt, thereby informing management and conservation decisions.

## STUDY AREA

Lake Roosevelt is located in northeast Washington. The reservoir was formed after the construction of Grand Coulee Dam on the Columbia River. Lake Roosevelt is 1–3 km wide, has a maximum depth of 122 m, and extends 241 km upstream from Grand Coulee Dam to the Canadian border (Polacek et al. 2006). The reservoir supports many recreational fisheries, including a Burbot fishery that may be underutilized and able to support greater harvest (Confederated Tribes of the Colville Reservation 2018).

## METHODS

*Fish collection and maintenance.*— Burbot were collected from Lake Roosevelt in November 2016 and March, June, and October 2017 by the Confederated Tribes of the Colville Reservation (Table 1). Baited cod traps were set shallower than 10 m to prevent barotrauma in captured fish and were retrieved the following day. Each fish was tagged with a PIT tag and transferred to the Bozeman Fish Technology Center, Bozeman, Montana. Fish were kept in four 485-L tanks ($244 \times 56 \times 36$ cm) at a stocking density of 43.2 g/L. Fish were maintained under a natural light cycle and a thermal profile ranging from 3.8°C to 16.7°C to match the thermal profile of Lake Roosevelt (Table 1; U.S. Bureau of Reclamation: https://www.usbr.gov/pn/hydromet/arcread.html). Live Rainbow Trout (150 mm) were constantly available as feed.

*Biological sampling.*— Biological data were collected monthly from 6–16 fish (Table 1) and included the following: body weight (±0.01 g), gonad weight (±0.01 g), TL (±0.01 cm), girth at the urogenital pore (±0.01 cm), blood, gonadal sonograms, and gonadal tissue. Fish were anesthetized with a dose of tricaine methanesulfonate (MS-222; 50 mg/L) for blood collection and were euthanized via an overdose of MS-222 (500 mg/L) for additional sample collection.

*Histology.*— Gonadal tissue was preserved in 10% phosphate-buffered formalin, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin or periodic acid–Schiff for histological analysis (Webb and Erickson 2007). The periodic acid–Schiff stain was used to highlight postovulatory follicles. Slides were examined under a Leica DM compound scope (250–450×; Leica Biosystems, Buffalo Grove, Illinois). Gametogenesis and reproductive condition in Burbot were described following the methods of Brown-Peterson et al. (2011) and Grier et al. (2009; Table 2).

*Gonadosomatic index and ovarian follicle diameter.*— Gonads were excised and weighed (±0.01 g) for calculation of the GSI ([gonad weight/total body weight] × 100). Ovarian follicles were collected from excised ovaries and preserved in Ringer's solution (Dettlaff et al. 1993). The diameters of 15 ovarian follicles (Johnson 1971) from each female were measured using image analysis (SPOT Imaging, Sterling Heights, Michigan).

*Plasma sex steroids.*— Blood was collected from the caudal vasculature using a heparinized syringe (3 $cm^3$ with 22-gauge needle; Webb et al. 2002). Plasma was separated by centrifugation ($1{,}228 \times g$ for 5 min) and stored at −80°C until analysis. Testosterone and E2 were extracted from the plasma following the methods of Fitzpatrick et al. (1987). Briefly, steroids from 100 µL of plasma were extracted twice with 2 mL of diethyl ether. Tubes were vortexed vigorously with ether, and the aqueous phase was removed by snap-freezing in liquid nitrogen. Extracted steroids were resuspended in 1 mL of phosphate-buffered saline with gelatin. Extraction recovery efficiencies for T and E2 were determined by adding tritiated steroids to tubes containing pooled plasma ($n = 4$). Extraction recovery efficiencies were 87–94% for T and 80–94% for E2. All steroid concentrations were corrected for the extraction recovery efficiency.

Plasma T and E2 concentrations were quantified by radioimmunoassay as described by Fitzpatrick et al. (1986) and as modified by Feist et al. (1990). Samples were assayed in duplicate. Quantified plasma sex steroid concentrations below the minimum quantifiable concentration (MQC) of the radioimmunoassay were assigned the MQC (0.27–0.37 ng/mL for T; 0.15–0.19 ng/mL for E2; concentrations varied due to extraction recovery efficiency). Nondetectable plasma sex steroid concentrations (i.e., not quantifiable) were assigned half of the MQC for statistical purposes (0.12–0.19 ng/mL for T; 0.06–0.09 ng/mL for E2; Croghan and Egeghy 2003). The intra-assay and interassay coefficients of variation for all assays were less than 5% and 10%, respectively.

Plasma 11-KT concentrations were quantified by liquid chromatography–mass spectrometry as described by M.-Z. Nouri, K. Kroll, and N. Denslow (University of Florida, unpublished data); the analysis took place in the laboratory of Nancy Denslow at the University of Florida, Gainesville. Plasma 11-KT concentrations below the MQC of the liquid chromatography–mass spectrometry analysis were assigned the MQC (0.02 ng/mL). Nondetectable 11-KT concentrations were assigned half of the MQC for statistical purposes (0.01 ng/mL; Croghan and Egeghy 2003). The accuracy of using plasma sex steroid concentrations to assign sex and reproductive condition was assessed using paired gonadal histology and plasma sex steroid concentrations.

TABLE 1. Numbers of Burbot that were collected from Lake Roosevelt, Washington, and sampled each month at the Bozeman Fish Technology Center. Burbot were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt. Data for monthly water temperatures (°C) are means. Data for body weight (g) and TL (cm) are reported as mean ± SD.

| Month | Number of fish collected from Lake Roosevelt | Number of fish sampled | Females sampled | Males sampled | Water temperature (°C) | Female body weight (g) | Male body weight (g) | Female TL (cm) | Male TL (cm) |
|---|---|---|---|---|---|---|---|---|---|
| Nov 2016 | 76 | 11 | 6 | 5 | 4.6 | 775.7 ± 251.9 | 653.7 ± 147.7 | 54.5 ± 54.5 | 49.3 ± 8.3 |
| Dec 2016 | | 11 | 6 | 5 | 4.0 | 940.78 ± 232.9 | 776.4 ± 149.8 | 52.9 ± 52.9 | 50.7 ± 1.6 |
| Jan 2017 | | 11 | 5 | 6 | 3.8 | 1,004.3 ± 113.9 | 903.3 ± 161.7 | 50.9 ± 1.7 | 50.7 ± 3.6 |
| Feb 2017 | | 12 | 6 | 6 | 3.8 | 955.2 ± 184.3 | 781.0 ± 201.0 | 49.9 ± 3.1 | 47.1 ± 3.7 |
| Mar 2017 | 95 | 13 | 5 | 8 | 3.9 | 909.6 ± 183.9 | 1,176.5 ± 518.5 | 50.0 ± 3.3 | 54.3 ± 6.2 |
| Apr 2017 | | 12 | 8 | 4 | 6.3 | 828.1 ± 240.5 | 865.0 ± 387.7 | 52.9 ± 4.4 | 50.7 ± 4.4 |
| May 2017 | 43 | 12 | 10 | 2 | 10.3 | 783.5 ± 200.4 | 580.7 ± 168.0 | 51.6 ± 4.5 | 51.0 ± 4.6 |
| Jun 2017 | | 16 | 7 | 9 | 13.5 | 714.9 ± 64.9 | 806.6 ± 249.5 | 54.2 ± 2.7 | 55.3 ± 4.0 |
| Jul 2017 | | 12 | 8 | 4 | 14.8 | 708.6 ± 219.2 | 827.4 ± 193.1 | 52.2 ± 4.5 | 54.2 ± 5.9 |
| Aug 2017 | | 12 | 10 | 2 | 14.9 | 857.2 ± 271.1 | 677.9 ± 170.4 | 53.1 ± 4.3 | 52.8 ± 1.6 |
| Sep 2017 | | 9 | 6 | 3 | 16.7 | 921.9 ± 262.1 | 646.3 ± 143.5 | 54.8 ± 3.7 | 51.6 ± 3.1 |
| Oct 2017 | 64 | 7 | 6 | 1 | 14.8 | 808.7 ± 175.2 | 813.7 | 53.5 ± 4.4 | 52.4 |
| Nov 2017 | | 6 | 3 | 3 | 12.8 | 744.1 ± 105.4 | 769.3 ± 59.9 | 51.9 ± 0.8 | 56.0 ± 4.5 |
| Dec 2017 | | 6 | 3 | 3 | 10.2 | 825.9 ± 115.8 | 583.9 ± 123.1 | 51.7 ± 4.3 | 46.4 ± 3.8 |
| Jan 2018 | | 7 | 3 | 4 | 5.5 | 828.7 ± 189.4 | 960.4 ± 457.6 | 52.8 ± 3.6 | 54.1 ± 8.6 |
| Feb 2018 | | 6 | 3 | 3 | 4.0 | 1,013.5 ± 290.2 | 777.0 ± 236.7 | 52.1 ± 4.9 | 47.7 ± 4.2 |
| Mar 2018 | | 6 | 3 | 3 | 4.6 | 751.5 ± 79.2 | 1,047.2 ± 280.6 | 46.3 ± 2.8 | 52.0 ± 5.0 |

TABLE 2. Reproductive condition and stages of maturity identified from histological analysis of gonadal tissue from Burbot. Stage numbers are referenced in parentheses. Germ cells were scored for reproductive condition and stage of maturity using a modified protocol of Grier et al. (2009) and Brown-Peterson et al. (2011). No oogonial proliferation (stage 1) females were sampled during this study.

| Sex | Reproductive condition | Stage of maturity | Description |
|---|---|---|---|
| Female | Nonreproductive | Oogonial proliferation (1) | Oogonia and potentially a few primary growth oocytes |
| | Nonreproductive | Primary growth (2) | Primary growth oocytes and oogonia |
| | Reproductive | Cortical alveolar (3) | Cortical alveoli on periphery of ovarian follicles |
| | Reproductive | Early vitellogenic (4) | Yolk granules accumulating in periphery of ovarian follicles; cortical alveoli on periphery of ovarian follicles; one layer of zona radiata |
| | Reproductive | Mid-vitellogenic (5) | Yolk globules with a mean diameter of 7.93 μm (95% CI = 5.97–9.89 μm) accumulating toward center or may be present throughout ovarian follicles; few cortical alveoli on periphery of ovarian follicles; one layer of zona radiata |
| | Reproductive | Late vitellogenic (6) | Yolk fusing into larger globules with a mean diameter of 19.62 μm (95% CI = 14.55–24.69 μm) throughout ovarian follicle; one layer of zona radiata; central germinal vesicle (i.e., nucleus) |
| | Reproductive | Ripe (7) | Yolk fused but not completely coalesced in all ovarian follicles; offset germinal vesicle |
| | Nonreproductive | Postovulatory (8) | Postovulatory follicles present with primary growth oocytes |
| | Nonreproductive | Atretic (9) | >75% atretic ovarian follicles, no postovulatory follicles present with primary growth oocytes |
| Male | Nonreproductive | Spermatogonial proliferation (1) | Cysts contain only spermatogonia |
| | Reproductive | Early spermatogenic (2) | Cysts contain spermatogonia and spermatocytes |
| | Reproductive | Mid-spermatogenic (3) | Few cysts contain spermatogonia; >50% of the cysts contain spermatocytes and spermatids |
| | Reproductive | Ripe and initiating spermiation (4) | Cysts filled with spermatozoa; approximately 20% of cysts emptying of spermatozoa |
| | Reproductive | Mid-spermiation (5) | Cysts with reduced or residual spermatozoa; some cysts may be empty |
| | Nonreproductive | Postspermiation (6) | Cysts with residual spermatozoa and cysts empty of spermatozoa |

*Ultrasound.*— A SonoSite Edge ultrasound (SonoSite, Bothell, Washington) with a linear transducer (6–15 MHz) was used to examine gonadal morphology in euthanized fish. The "small parts" exam type was used, with the optimization set to "general." The ultrasound scanning depth was set between 1.8 and 2.2 cm. Fish were oriented ventrally, and gonadal sonograms were taken (1) with the transducer placed perpendicular to the midline at the urogenital pore, (2) with the transducer placed perpendicular to the midline and anterior to the urogenital pore, and (3) with the transducer placed parallel to the midline. The accuracy of using ultrasound to assign sex and reproductive condition was assessed using paired gonadal histology and gonadal sonograms.

*Data analyses.*— One-way ANOVA was used to test for a significant difference in GSI, ovarian follicle diameter, and plasma sex steroid concentrations among stages of maturity. The ANOVA model was modified for the underlying distribution to be the beta distribution when analyzing GSI data, which is bounded between 0 and 1 (Cribari-Neto and Zeileis 2010). Data were checked for nonnormality by examining the distribution of the model residuals. Ovarian follicle diameter, and plasma sex steroid concentrations were log transformed. The accepted significance level $\alpha$ was 0.05. Pairwise comparisons with a Bonferroni correction were used to test for significant differences in GSI, ovarian follicle diameter, and plasma sex steroid concentrations between stages of maturity.

AdaBoost algorithms were used to determine the accuracy of plasma sex steroid concentrations (T, E2, and 11-KT) for assigning sex and reproductive condition in Burbot (Alfaro et al. 2013). AdaBoost is a machine

learning algorithm that creates a highly accurate classification model by combining many weak models (Schapire 2013). Once the classification model has been trained, it can be used to make predictions about new data (Alfaro et al. 2013). The classification models were trained using sex and reproductive condition determined by gonadal histology and the corresponding plasma sex steroid concentrations. The classification models were then used to predict sex and reproductive condition within a sex by plasma sex steroid concentrations. The accuracy of prediction was determined by leave-one-out cross validation. Classification models were parameterized with all combinations of plasma sex steroids and girth at the urogenital pore (Table 3). We only used plasma sex steroid concentrations and girth at the urogenital pore for the predictive models because this study was focused on nonlethal tools to assign sex and reproductive condition in Burbot. Statistical analyses were completed using R version 3.3.2. Data and R code are included in the Supplementary Material available in the online version of this article.

TABLE 3. Classification model accuracy (%) of assigning sex and reproductive condition in adult Burbot. Burbot were held at the Bozeman Fish Technology Center and were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt, Washington (T = testosterone; E2 = estradiol-17β; 11-KT = 11-ketotestosterone; UGP Girth = girth at the urogenital pore).

| Assignment | Model | Accuracy (%) |
|---|---|---|
| Sex (Jun–May) | 11-KT | 82 |
| | T + E2 + 11-KT | 80 |
| | T + E2 | 78 |
| | T + 11-KT | 78 |
| | 11-KT + E2 | 78 |
| | E2 | 72 |
| | T | 64 |
| Sex (Nov–Mar) | E2 + 11-KT | 98 |
| | T + E2 + 11-KT | 98 |
| | E2 | 94 |
| | 11-KT | 94 |
| | T + 11-KT | 93 |
| | T + E2 | 91 |
| | T | 60 |
| Female reproductive condition | T + E2 | 98 |
| | T | 97 |
| | T + UGP Girth | 97 |
| | T + E2 + UGP Girth | 97 |
| | E2 | 91 |
| | E2 + UGP Girth | 89 |
| | UGP Girth | 66 |
| Male reproductive condition | T | 90 |
| | T + 11-KT | 90 |
| | T + 11-KT + UGP Girth | 88 |
| | 11-KT | 87 |
| | T + UGP Girth | 86 |
| | 11-KT + UGP Girth | 85 |
| | UPG Girth | 79 |

## RESULTS

### Histology

Gametogenesis was described by gonadal histology during an entire reproductive cycle (Table 2; Figures 1, 2). Reproductive condition was characterized by the stages of maturity indicating that a fish was reproductive (i.e., had initiated gonadal development to spawn or was capable of spawning) or nonreproductive (i.e., had not initiated gonadal development to spawn or had already spawned; Brown-Peterson et al. 2011).

### Gonadosomatic Index and Ovarian Follicle Diameter

Female GSI differed significantly among stages of maturity ($F = 192.65$, df = 7, $P < 0.001$). Mean female GSI increased from 0.79 (95% CI = 0.71–0.87) during primary growth (stage 2) to 13.19 (95% CI = 9.67–16.71) when ovarian follicles were ripe (stage 7) and then decreased to 0.94 (95% CI = 0.74–1.15) during postovulation (stage 8; Figure 3). Female GSI was highly variable during atresia (stage 9), ranging from 2.39 to 13.64, because atresia was more advanced in some females than in others (Figure 3).

Male GSI differed significantly among stages of maturity ($F = 163.78$, df = 5, $P < 0.001$). Mean male GSI increased from 0.38 (95% CI = 0.28–0.47) during spermatogonial proliferation (stage 1) to 16.16 (95% CI = 13.42–18.89) during mid-spermatogenesis (stage 3) and steadily decreased to 1.14 (95% CI = 0.15–2.12) during postspermiation (stage 6; Figure 3).

Ovarian follicle diameter differed significantly among stages of maturity ($F = 493.39$; df = 6, 71; $P < 0.001$). Mean ovarian follicle diameter increased from 155 μm (95% CI = 146–165 μm) during primary growth (stage 2) to 859 μm (95% CI = 776–942 μm) when ovarian follicles were ripe (stage 7; Figure 3). Some atretic (stage 9) ovarian follicles were extremely deformed and could not be accurately measured; therefore, atretic ovarian follicles were removed from the analysis.

### Plasma Sex Steroids

Female plasma T concentration differed significantly among stages of maturity ($F = 169.35$; df = 7, 90; $P < 0.001$). Mean female plasma T concentration increased from 0.18 ng/mL (95% CI = 0.16–0.21 ng/mL) during primary growth (stage 2) to 24.36 ng/mL (95% CI = 19.17–29.56 ng/mL) during late vitellogenesis (stage 6; Table 4). Mean female plasma T concentration remained elevated

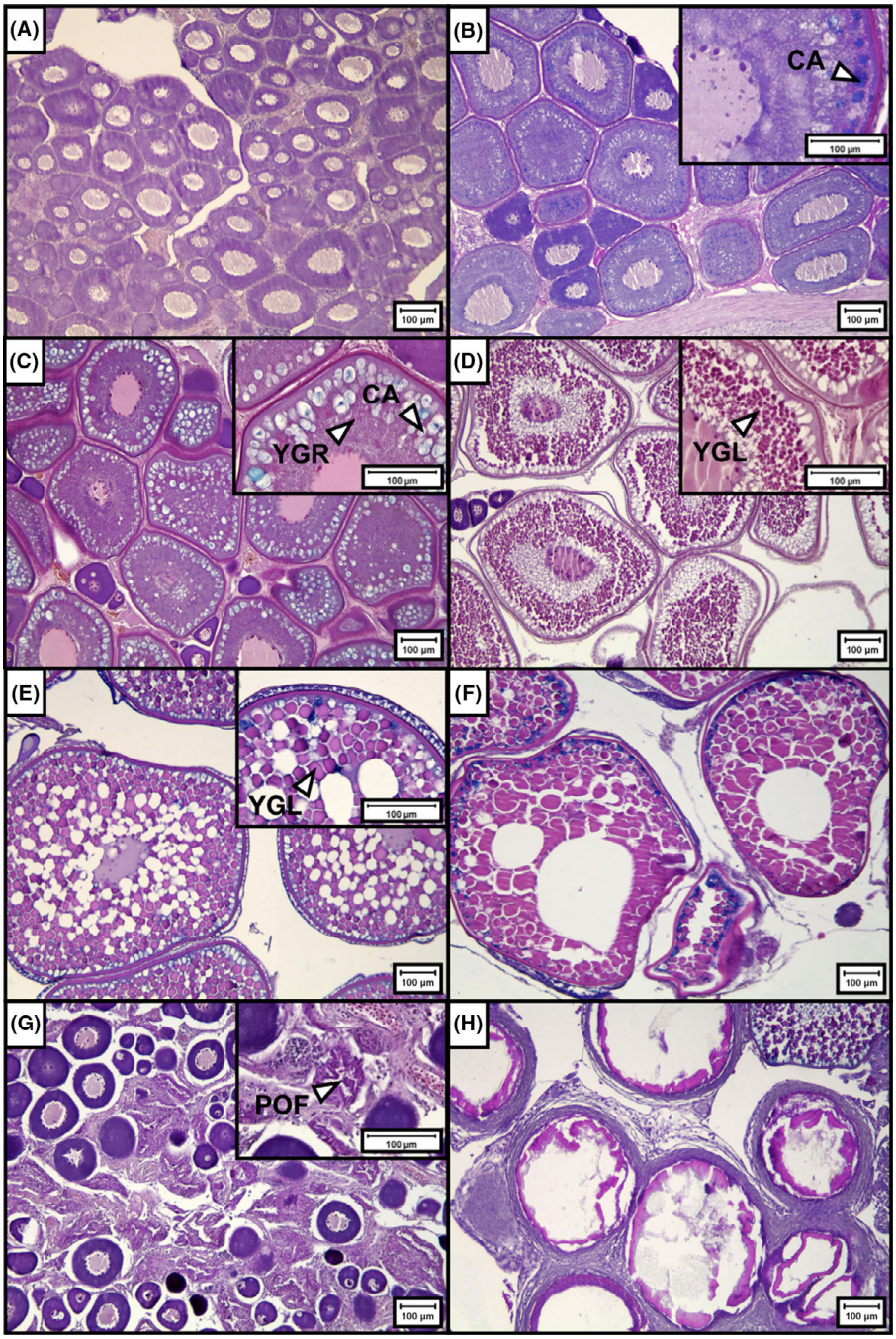


FIGURE 1. Histological description of gametogenesis in female Burbot: **(A)** primary growth (stage 2) oocytes with oogonia (hematoxylin and eosin stain [H&E]); **(B)** cortical alveolar (stage 3) ovarian follicles with cortical alveoli (CA) on periphery (periodic acid–Schiff stain [PAS]); **(C)** early vitellogenic (stage 4) ovarian follicles accumulating yolk granules (YGR) and CA on periphery (H&E); **(D)** mid-vitellogenic (stage 5) ovarian follicles with yolk globules (YGL; mean diameter = 7.69 µm; 95% CI = 5.97–9.89 µm) accumulating toward center and few CA on periphery (H&E); **(E)** late vitellogenic (stage 6) ovarian follicles with YGL (mean diameter = 19.62 µm; 95% CI = 14.55–24.69 µm) throughout (PAS); **(F)** ripe (stage 7) ovarian follicles with fused yolk (PAS); **(G)** postovulatory (stage 8) follicles (POF) with primary growth oocytes (PAS); and **(H)** atretic (stage 9) follicles (PAS).

when ovarian follicles were ripe (stage 7) and then decreased to nondetectable during postovulation (stage 8; Table 4). Female plasma T concentration was variable during atresia (stage 9), ranging from nondetectable to 11.76 ng/mL (Table 4). Female plasma E2 concentration differed significantly among stages of maturity ($F =$

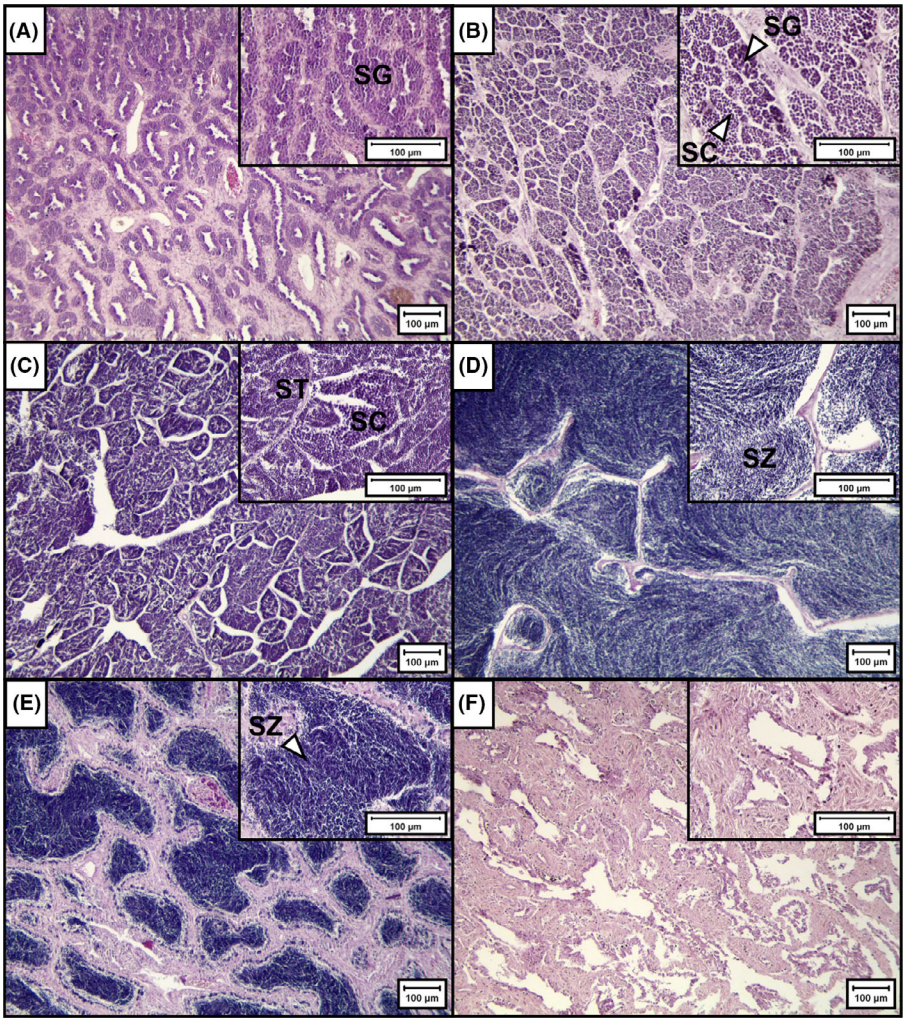


FIGURE 2. Histological description of gametogenesis in male Burbot: **(A)** spermatogonial proliferation (stage 1) cysts with only spermatogonia (SG; periodic acid–Schiff stain [PAS]); **(B)** early spermatogenic (stage 2) cysts with spermatocytes (SC) and SG (hematoxylin and eosin stain [H&E]); **(C)** mid-spermatogenic (stage 3) cysts with spermatids (ST) and SC (and with SG potentially also present; H&E); **(D)** ripe (stage 4) cysts filled with spermatozoa (SZ; H&E); **(E)** mid-spermiation (stage 5) cysts with reduced SZ (PAS); and **(F)** postspermiation (stage 6) cysts empty of SZ (PAS).

133.04; df = 7, 90; $P < 0.001$). Mean female plasma E2 concentration increased from nondetectable when cortical alveoli appeared (stage 3) to 7.56 ng/mL (95% CI = 5.54–9.57 ng/mL) during late vitellogenesis (stage 6) and then decreased to 0.23 ng/mL (95% CI = 0.00–0.92 ng/mL) when ovarian follicles were ripe (stage 7; Table 4). Female plasma E2 concentration was variable during atresia (stage 9), ranging from nondetectable to 2.8 ng/mL (Table 4). Female plasma 11-KT concentration varied from nondetectable to 0.18 ng/mL among all stages of maturity. Female plasma 11-KT concentration is not reported further because of the nondetectable or low values among all stages of maturity.

Male plasma T concentration differed significantly among stages of maturity ($F = 63.30$; df = 5, 65; $P < 0.001$). Mean male plasma T concentration increased from 0.28 ng/mL (95% CI = 0.12–0.44 ng/mL) during spermatogonial proliferation (stage 1) to 23.78 ng/mL (95% CI = 16.78–30.78 ng/mL) when testes were ripe (stage 4) and then decreased to 0.31 ng/mL (95% CI = 0.00–0.80 ng/mL) after spermiation (stage 6; Table 5). Male plasma 11-KT concentration differed significantly among stages of maturity ($F = 23.02$; df = 5, 61; $P < 0.001$). Mean plasma 11-KT concentration increased from 0.07 ng/mL (95% CI = 0.01–0.13 ng/mL) during spermatogonial proliferation (stage 1) to 28.90 ng/mL (95% CI = 8.53–49.27 ng/mL) when testes were ripe (stage 4) and then decreased to 0.26 ng/mL (95% CI = 0.00–0.79 ng/mL) after spermiation (stage 6; Table 5). Male plasma E2 concentration varied from nondetectable to 0.70 ng/mL among all stages of maturity. Male plasma E2 concentration is not reported further because of the nondetectable or low values among all stages of maturity.

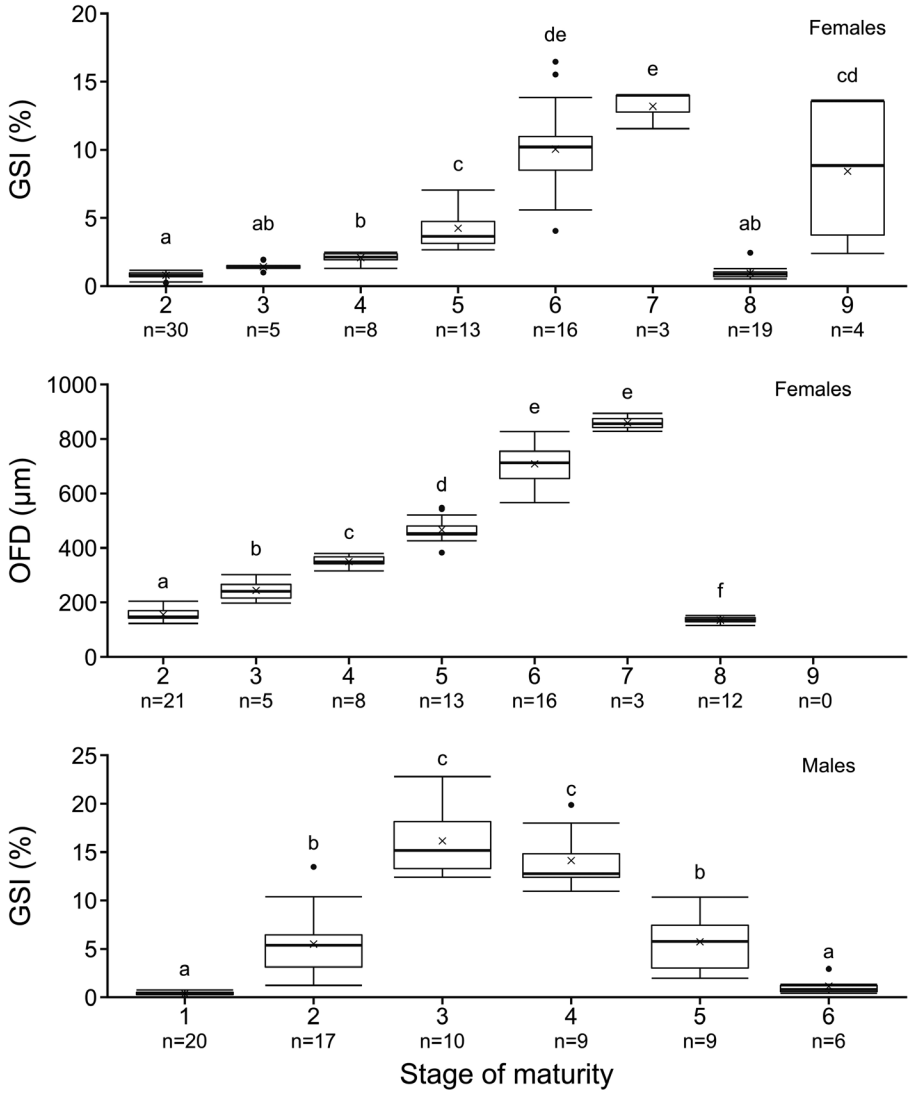


FIGURE 3. Gonadosomatic index (GSI; %) of adult female Burbot, ovarian follicle diameter (OFD; µm) of adult female Burbot, and GSI of adult male Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt, Washington. Data are the observed values of GSI and OFD. No females were observed during oogonial proliferation (stage 1). The box encompasses data from the first to third quartiles (i.e., 25th to 75th percentiles); the horizontal line within each box represents the median; the × -symbol represents the mean; and the whiskers represent the minimum and maximum values. Outliers are represented by individual points. If there are outliers, the whiskers represent the minimum value within 1.5 times the interquartile range below the first quartile or the maximum value within 1.5 times the interquartile range above the third quartile. Different letters among stages of maturity indicate significant differences. Stages of maturity are defined in Table 2.

Plasma 11-KT concentration was the best predictor of sex during the entire reproductive cycle, with 97% accuracy in females and 61% accuracy in males (Table 6). The combination of plasma 11-KT and plasma E2 concentrations was the best predictor of sex when fish were reproductive (i.e., November–March in Lake Roosevelt), with 98% accuracy in both females and males (Table 6).

The combination of plasma T and plasma E2 concentrations was the best predictor of reproductive condition in females, with 97% accuracy in nonreproductive females and 100% accuracy in reproductive females (Table 7). Plasma T concentration was the best predictor of reproductive condition in males, with 92% accuracy in nonreproductive males and 89% accuracy in reproductive males (Table 7).

## Ultrasound

Distinct morphological characteristics were observed between ovaries and testes in Burbot. Lobes of the ovaries were rounded and connected at the urogenital

TABLE 4. Plasma testosterone (T) and estradiol-17β (E2) concentrations (ng/mL) in adult female Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt, Washington. Data were collected from November 2016 to March 2018. Data are presented as mean ± SD, with sample size in parentheses. Quantified plasma sex steroid concentrations below the minimum quantifiable concentration (MQC) of the radioimmunoassay were assigned the MQC (T: 0.27–0.32 ng/mL; E2: 0.15–0.17 ng/mL). Nondetectable plasma sex steroid concentrations were assigned half of the MQC (T: 0.12–0.19 ng/mL; E2: 0.06–0.09 ng/mL). No females were observed during oogonial proliferation (stage 1). Within a given column, different letters indicate significant differences in the specified steroid among stages of maturity.

| Stage of maturity | T | E2 |
|---|---|---|
| Primary growth (stage 2) | 0.18 ± 0.07 zy ($n = 30$) | 0.10 ± 0.11 z ($n = 30$) |
| Cortical alveolar (stage 3) | 0.45 ± 0.22 zx ($n = 5$) | 0.20 ± 0.24 z ($n = 5$) |
| Early vitellogenic (stage 4) | 1.39 ± 0.40 w ($n = 8$) | 0.78 ± 0.28 y ($n = 8$) |
| Mid-vitellogenic (stage 5) | 4.50 ± 2.15 v ($n = 13$) | 4.40 ± 2.57 x ($n = 13$) |
| Late vitellogenic (stage 6) | 24.36 ± 9.75 u ($n = 16$) | 7.56 ± 3.78 x ($n = 16$) |
| Ripe (stage 7) | 23.14 ± 17.28 u ($n = 3$) | 0.23 ± 0.28 z ($n = 3$) |
| Postovulatory (stage 8) | 0.13 ± 0.03 y ($n = 19$) | 0.08 ± 0.01 z ($n = 19$) |
| Atretic (stage 9) | 3.16 ± 5.74 xw ($n = 4$) | 0.76 ± 1.36 z ($n = 4$) |

TABLE 5. Plasma testosterone (T) and 11-ketotestosterone (11-KT) concentrations (ng/mL) in adult male Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt, Washington. Data were collected from November 2016 to March 2018. Data are presented as mean ± SD, with sample size in parentheses. Quantified plasma sex steroid concentrations below the minimum quantifiable concentration (MQC) of the radioimmunoassay were assigned the MQC (T: 0.27–0.37 ng/mL; 11-KT: 0.02 ng/mL). Nondetectable plasma sex steroid concentrations were assigned half of the MCQ (T: 0.11–0.16 ng/mL; 11-KT: 0.01 ng/mL). Within a given column, different letters indicate significant differences in the specified steroid among stages of maturity.

| Stage of maturity | T | 11-KT |
|---|---|---|
| Spermatogonial proliferation (stage 1) | 0.28 ± 0.35 z ($n = 20$) | 0.07 ± 0.13 z ($n = 20$) |
| Early spermatogenic (stage 2) | 5.91 ± 2.64 y ($n = 17$) | 3.36 ± 2.95 y ($n = 15$) |
| Mid-spermatogenic (stage 3) | 19.54 ± 6.24 x ($n = 10$) | 9.78 ± 4.70 y ($n = 8$) |
| Ripe (stage 4) | 23.78 ± 9.10 x ($n = 9$) | 28.90 ± 26.50 y ($n = 9$) |
| Mid-spermiation (stage 5) | 3.55 ± 3.58 w ($n = 9$) | 3.64 ± 3.65 y ($n = 9$) |
| Postspermiation (stage 6) | 0.31 ± 0.47 z ($n = 6$) | 0.26 ± 0.50 z ($n = 6$) |

pore (Figure 4). Testes were angular and unconnected at the urogenital pore (Figure 4). The morphological differences between ovaries and testes were easily delineated using ultrasound when fish were reproductive. It was more difficult to differentiate ovaries and testes via ultrasound when fish were nonreproductive (Figure 5).

Sex was assigned with close to 100% accuracy in females by using ultrasound (Table 8). Only a single female was misclassified during primary growth (stage 2). Sex was assigned with 91% accuracy in males by using ultrasound (Table 8). Only two males were misclassified during spermatogonial proliferation (stage 1), one was misclassified during mid-spermatogenesis (stage 3), and two were misclassified during postspermiation (stage 6).

Ultrasound was not useful for assigning reproductive condition during this study. Morphological and echogenic differences among gonads at differing stages of maturity could not be delineated using the SonoSite Edge ultrasound unit. For example, ovarian follicles could not be delineated because they were smaller than the resolution of the ultrasound transducer (1 mm).

## DISCUSSION

To our knowledge, this study represents the first complete histological description of gametogenesis in Burbot. A partial description of gametogenesis in Burbot has already been published (Schaefer et al. 2016), but that description only included the previtellogenic and vitellogenic stages of maturity in females and the spermatogenic stages of maturity in males. In addition to the previtellogenic, vitellogenic, and spermatogenic stages of maturity, our description of gametogenesis includes the postovulation and atretic stages of maturity in females and the spermatogonial proliferation and postspermiation stages of maturity in males. A complete description of gametogenesis by gonadal histology enabled us to validate nonlethal tools (plasma sex steroid concentrations and ultrasound) for assigning sex and reproductive condition.

The pattern of sex steroid synthesis in fish (i.e., plasma sex steroid profiles) enables the successful assignment of sex and reproductive condition (e.g., Ceapa et al. 2002; Webb et al. 2002; Feist et al. 2004; Viayeh et al. 2006; Webb and Erickson 2007; Chu-Koo et al. 2009; Craig et al. 2009; Kohn et al. 2013; Bangs and Nagler 2014; Grieshaber et al. 2016; Du et al. 2017). In Burbot, plasma 11-KT concentration assigned sex with 82% accuracy during the entire year and the combination of plasma 11-KT and E2 concentrations assigned sex with 98% accuracy when fish were reproductive (i.e., November–March in

TABLE 6. Accuracy of assigning sex in adult Burbot by using an AdaBoost algorithm. Burbot were held at the Bozeman Fish Technology Center and were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt, Washington. Plasma 11-ketotestosterone (11-KT) concentration was used as a predictor during the entire reproductive cycle (June–May in Lake Roosevelt), and the combination of plasma 11-KT and plasma estradiol-17β (E2) concentrations was used as a predictor when fish were reproductive (November–March in Lake Roosevelt). Values are the percentages of fish that were correctly assigned (in bold) or incorrectly assigned (in italics), with sample size in parentheses.

| | Predicted | | |
|---|---|---|---|
| Actual | Female | Male | Total |
| | **Jun–May** | | |
| Female | **97** ($n = 88$) | *3* ($n = 3$) | 100 ($n = 91$) |
| Male | *39* ($n = 26$) | **61** ($n = 41$) | 100 ($n = 67$) |
| | **Nov–Mar** | | |
| Female | **98** ($n = 41$) | *2* ($n = 1$) | 100 ($n = 42$) |
| Male | *2* ($n = 1$) | **98** ($n = 41$) | 100 ($n = 42$) |

Lake Roosevelt). Plasma 11-KT and E2 concentrations have been used to successfully assign sex in other fishes, such as the Stellate Sturgeon (Ceapa et al. 2002), Arapaima (Chu-Koo et al. 2009), and Hapuka (Kohn et al. 2013). Plasma 11-KT and E2 concentrations were used to assign sex in reproductive Hapuka (i.e., 2 months prior to the spawning season and during the spawning season) with 100% accuracy but were not useful in nonreproductive Hapuka (i.e., postspawn; Kohn et al. 2013). We also observed a lower accuracy of assigning sex using plasma 11-KT and E2 concentrations in nonreproductive Burbot. Plasma sex steroids are not synthesized until fish have initiated gonadal development to spawn (Feist et al. 2004). In females, plasma T and E2 concentrations increase during vitellogenesis (i.e., accumulation of yolk in ovarian follicles; Feist et al. 2004; Kagawa 2013; Wootton and Smith 2015:46–80). Plasma 11-KT concentrations may also increase during vitellogenesis depending on the species (Borg 1994). In males, plasma T and 11-KT concentrations increase with the initiation of spermatogenesis (i.e., meiosis), while plasma E2 concentrations remain low (Feist et al. 2004; Pankhurst 2008; Schulz et al. 2010; Wootton and Smith 2015:46–80). Both sexes have low plasma sex steroid concentrations before the initiation of a reproductive cycle and after spawning until initiation of the next reproductive cycle (Pankhurst 2008), making it difficult to assign sex using plasma sex steroid concentrations when fish are nonreproductive. Sampling time is an important consideration when assigning sex and reproductive condition in Burbot. To provide the highest accuracy of assignment, plasma samples should be collected when fish are reproductive and sex steroid concentrations are elevated (i.e., November–March in Lake Roosevelt). Plasma samples should also be collected before spawning because sex steroid concentrations cannot be used to reliably differentiate between immature fish and postspawn fish.

In female Burbot, plasma T and E2 concentrations were used to assign reproductive condition (nonreproductive or reproductive) with 98% accuracy. Plasma 11-KT concentration was not used to assign reproductive condition because it was nondetectable or low in all females, as has been observed in other teleost species (Borg 1994). In male Burbot, plasma T concentration was used to assign reproductive condition with 90% accuracy; plasma 11-KT concentration may also be used to assign reproductive condition with 87% accuracy. Plasma sex steroid concentrations have been used to assign reproductive condition in other fishes, such as the White Sturgeon (Webb et al. 2002), Persian Sturgeon (Viayeh et al. 2006), Green Sturgeon *Acipenser medirostris* (Webb and Erickson 2007), and Cutthroat Trout (Bangs and Nagler 2014). Assignment of reproductive condition can be used to determine the proportion of females and males that spawn during an annual reproductive cycle, which has been documented to vary among Burbot populations (Pulliainen and Korhonen

TABLE 7. Accuracy of assigning reproductive condition in adult female and male Burbot by using an AdaBoost algorithm. Burbot were held at the Bozeman Fish Technology Center and were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt, Washington. In females, the combination of plasma testosterone (T) and plasma estradiol-17β (E2) concentrations was used as a predictor. In males, plasma T concentration was used as a predictor. Values are the percentages of fish that were correctly assigned (in bold) or incorrectly assigned (in italics), with sample size in parentheses.

| | Predicted | | |
|---|---|---|---|
| Actual | Nonreproductive | Reproductive | Total |
| | **Females** | | |
| Nonreproductive | **97** ($n = 56$) | *3* ($n = 2$) | 100 ($n = 58$) |
| Reproductive | *0* ($n = 0$) | **100** ($n = 40$) | 100 ($n = 40$) |
| | **Males** | | |
| Nonreproductive | **92** ($n = 24$) | *8* ($n = 2$) | 100 ($n = 26$) |
| Reproductive | *11* ($n = 5$) | **89** ($n = 40$) | 100 ($n = 45$) |

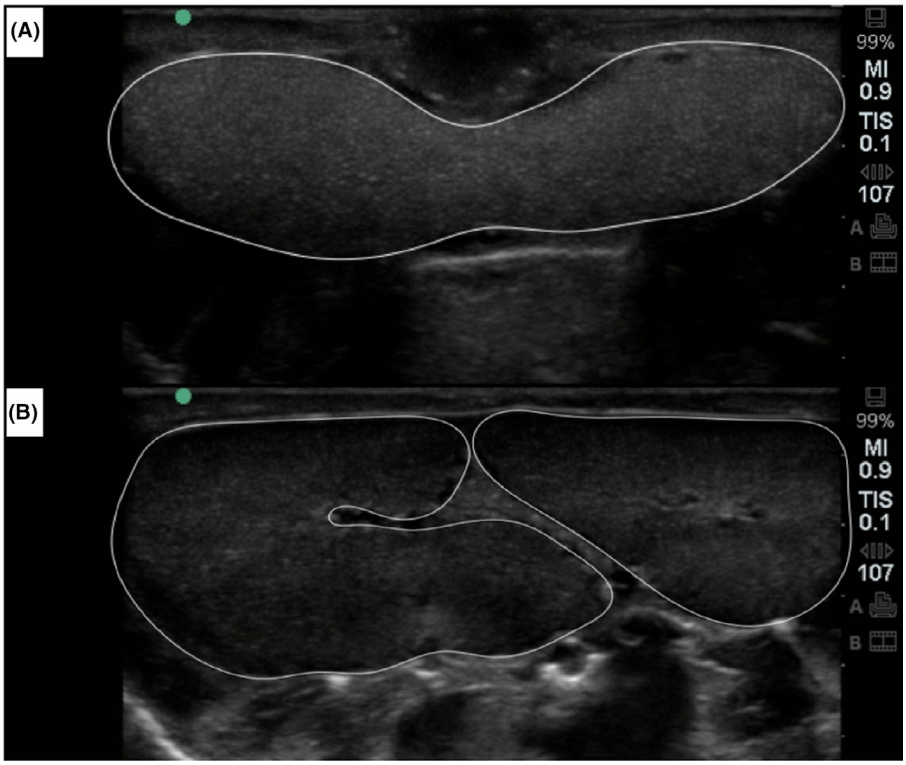


FIGURE 4. Sonograms of **(A)** late vitellogenic (stage 6) ovarian tissue from an adult female Burbot and **(B)** ripe (stage 4) testicular tissue from an adult male Burbot in February. The ultrasound transducer was placed perpendicular to the midline at the urogenital pore to capture the images. The white lines delineate ovarian tissue (panel A) and testicular tissue (panel B). The lobes of the ovaries were rounded and clearly connected (panel A), whereas the testes were angular and unconnected (panel B). [Color figure can be viewed at afsjournals.org.]

1990; McPhail and Paragamian 2000). For example, approximately 30% of adult Burbot in Bothnian Bay, Finland, were not developing to spawn during an annual reproductive cycle (Pulliainen and Korhonen 1990). Variation in the proportion of fish that spawn may negatively affect the reproductive potential of a population, and accounting for this variation may influence management decisions (Morgan 2008; Rideout and Tomkiewicz 2011).

Plasma T did not improve the accuracy of assigning sex in Burbot but was useful in assigning reproductive condition. The combination of plasma 11-KT and E2 concentrations was the best predictor of sex. In teleosts, 11-KT is considered the primary androgen (Borg 1994), and plasma 11-KT and E2 concentrations have been used to successfully assign sex in other teleosts (Chu-Koo et al. 2009; Kohn et al. 2013). The combination of plasma T and E2 concentrations may be used to assign sex in Burbot but with a lower accuracy. Using plasma T and E2 concentrations to assign sex may be preferable because only two steroids would need to be analyzed to assign sex and reproductive condition, which may reduce the cost of analyzing samples. The decision of which plasma sex steroids to analyze will depend on the specific objectives and limitations of a fisheries program, such as acceptable levels of assignment accuracy and funding.

The plasma sex steroid profiles described in this study are similar to the profiles described for other teleosts with synchronous gonadal development, such as Rainbow Trout (Scott et al. 1980a, 1980b), Coho Salmon *O. kisutch* (Fitzpatrick et al. 1986), and Masu Salmon *O. masou* (Kagawa 2013). However, the plasma sex steroid profiles we observed differed from those previously observed in Burbot by Mustonen et al. (2002). Unlike our observations, plasma E2 concentrations remained elevated in ripe (mean = 7.16 ng/mL) and postovulatory (mean = 5.36 ng/mL) female Burbot, and plasma T concentrations were much lower in ripe male Burbot (mean = 10.38 ng/mL; Mustonen et al. 2002). Plasma sex steroid concentrations may differ among populations because environmental contaminants are known to disrupt the synthesis of sex steroids (Kime 1995; Nicolas 1999; Feist et al. 2005; Hinck et al. 2008). The classification models described in this paper can be applied to other Burbot populations; however, plasma sex steroid concentrations should first be quantified in a subset of the naïve population to determine whether the plasma sex steroid profiles are similar to those observed in the Lake Roosevelt population.

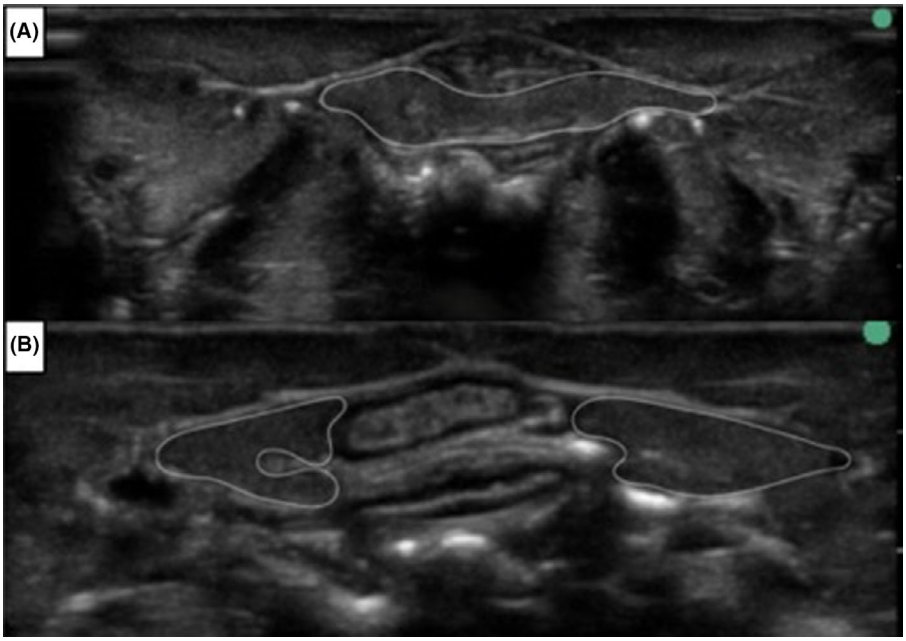


FIGURE 5. Sonograms of **(A)** primary growth (stage 2) ovarian tissue from an adult female Burbot and **(B)** spermatogonial proliferation (stage 1) testicular tissue from an adult male Burbot in June. The ultrasound transducer was placed perpendicular to the midline at the urogenital pore to capture the images. The white lines delineate ovarian tissue (panel A) and testicular tissue (panel B). Ovaries were undeveloped and had an appearance similar to that of testes. The lobes of the ovaries were clearly connected, whereas the testes were unconnected. [Color figure can be viewed at afsjournals.org.]

TABLE 8. Accuracy of assigning sex using ultrasound in adult Burbot by stage of maturity. Burbot were held at the Bozeman Fish Technology Center and were exposed to a natural photoperiod and thermal profile similar to that of Lake Roosevelt, Washington. The percentage of fish correctly assigned is reported, with sample size (*n*) in parentheses. Data collected from November 2016 are not included because that month was used as a training month. No females were sampled during oogonial proliferation (stage 1). Stages 7–9 were not described in males. See Table 2 for descriptions of the stages of maturity.

| | Ultrasound accuracy (%) | |
|---|---|---|
| Stage of maturity | Females | Males |
| 1 | NA | 90 (20) |
| 2 | 97 (30) | 100 (12) |
| 3 | 100 (5) | 90 (10) |
| 4 | 100 (3) | 100 (9) |
| 5 | 100 (12) | 100 (9) |
| 6 | 100 (16) | 67 (6) |
| 7 | 100 (3) | NA |
| 8 | 100 (19) | NA |
| 9 | 100 (4) | NA |
| Mean | 100 | 91 |

Ultrasound was used to assign sex with close to 100% accuracy in female Burbot and 91% accuracy in male Burbot by detecting differences in gonadal morphology. Many studies have previously used ultrasound to accurately assign sex in fishes (e.g., Karlsen and Holm 1994; Moghim et al. 2002; Newman et al. 2008; Loher and Stephens 2011; Kohn et al. 2013; Bangs and Nagler 2014). Sex was assigned in reproductive Atlantic Cod (a member of the same taxonomic order as Burbot) with 95% accuracy using ultrasound to detect echogenic differences between ovaries and testes (Karlsen and Holm 1994). Sex was more difficult to assign in nonreproductive Atlantic Cod because there was little echogenic difference between ovaries and testes (Karlsen and Holm 1994). More recently, sex was assigned in nonreproductive and reproductive Pacific Halibut by using ultrasound to detect morphological differences between ovaries and testes, with 100% accuracy (Loher and Stephens 2011). In Pacific Halibut, ovaries are rounded and tapered, whereas testes are sickle-shaped and pointed (Loher and Stephens 2011). We were also able to assign sex in both nonreproductive and reproductive Burbot by using morphological differences between ovaries and testes, although accuracy decreased when gonads were small and undeveloped (e.g., 67% in postspawn males). Therefore, sampling time should also be considered when using ultrasound to assign sex in Burbot.

In this study, reproductive condition could not be assigned using ultrasound because ovarian follicles (<1 mm in diameter) were too small to be delineated with the SonoSite Edge ultrasound unit. Ultrasound has been

used to assign reproductive condition in other female teleosts, such as steelhead and Channel Catfish, by detecting ovarian follicles (Evans et al. 2004; Novelo and Tiersch 2016). Compared to the ovarian follicles we observed in ripe female Burbot, ovarian follicles in ripe female steelhead are much larger (3 mm in diameter), allowing them to be easily delineated using ultrasound (Evans et al. 2004). Other methods, such as employing ultrasound to measure gonad size, may be used to assign reproductive condition in Burbot because GSI and ovarian follicle diameter significantly differed among stages of maturity (McGarvey 2019).

In conclusion, plasma sex steroids can be used to assign sex and reproductive condition in Burbot with high accuracy. Ultrasound can also be used to accurately assign sex in Burbot and should be further evaluated to assign reproductive condition. The nonlethal tools assessed in this paper will enable fisheries biologists to assess reproductive indices of the Burbot population in Lake Roosevelt and have the potential to be applied to other Burbot populations. Assessing reproductive indices will provide fisheries biologists with essential information to improve management decisions.

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## SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.